Analysis of lipids and polycyclic aromatic hydrocarbons as indicators of past and present (micro)biological activity

Wiesenberg, Guido L B ; Gocke, Martina

Abstract: Analysis of lipids and hydrocarbons is performed frequently in recent and ancient plant tissues, soils, sediments, peat deposits, oil, rocks, anthropogenic artifacts (archeological samples), and other materials to trace the contribution of different biological and anthropogenic sources of organic matter as well as environmental changes and the fate of organic matter like degradation. The approaches for the analysis of lipids and hydrocarbons strongly vary from traditional methodologies like thin-layer chromatography to universal approaches like pyrolysis, whereas the preparative separation of lipid fractions based on their polarity enables gas-chromatographic analyses of single fractions and compound-specific analysis of stable (2H/1H, 13C/12C) and radioactive (14C) isotope compositions. Often, lipid extraction operationally defines a subfraction of total lipids. On the one hand, free extractable lipids are obtained by extraction with organic solvents, whereas on the other hand, total samples or extraction residues are extracted for more polar lipid fractions using highly polar organic solvents and water, to release bound lipids. Procedures for extraction of free extractable lipids are diverse and mainly defined by the target of research and availability of instrumentation. In the current protocol, state-of-the-art techniques for the investigation of free extract- able lipids in various materials are explained, which can be applied even in laboratory environments with limited technical equipment. The protocols cover sample preparation, extraction, purification, analysis, as well as a brief overview of the data evaluation using lipid molecular proxies and compound-specific isotopes.

DOI: https://doi.org/10.1007/8623_2015_157

Metadata of the chapter that will be visualized online

Chapter Title	Analysis of Lipids and Polycyclic Aromatic Hydrocarbons as Indicators of Past and Present (Micro)Biological Activity	
Copyright Year		
Copyright Holder	Springer-Verlag Berlin Heidelberg	
Corresponding Author	Family Name	**Wiesenberg**
	Particle	
	Given Name	**Guido L.B.**
	Suffix	
	Division	Department of Geography
	Organization	University of Zurich
	Address	Zurich, Switzerland
	Email	guido.wiesenberg@geo.uzh.ch
Author	Family Name	**Gocke**
	Particle	
	Given Name	**Martina I.**
	Suffix	
	Division	Department of Geography
	Organization	University of Zurich
	Address	Zurich, Switzerland
Abstract	Analysis of lipids and hydrocarbons was performed frequently in recent and ancient plant tissues, soils, sediments, peat deposits, oil, rocks, anthropogenic artifacts (archeological samples), and other materials to trace the contribution of different biological and anthropogenic sources of organic matter as well as environmental changes and the fate of organic matter like degradation. The approaches for the analysis of lipids and hydrocarbons strongly vary from traditional methodologies like thin-layer chromatography to universal approaches like pyrolysis, whereas the preparative separation of lipid fractions based on their polarity enables gas-chromatographic analyses of single fractions and compound-specific analysis of stable ($^{1}H/^{2}H$, $^{12}C/^{13}C$) and radioactive (^{14}C) isotope compositions. Often, lipid extraction operationally defines a subfraction of total lipids. On the one hand, free extractable lipids are obtained by extraction with organic solvents, whereas on the other hand, total samples or extraction residues are extracted for more polar lipid fractions using highly polar organic solvents and water, to release bound lipids. Also, procedures for extraction of free extractable lipids are diverse and mainly defined by the target of research and availability of instrumentation. In the current protocol, state-of-the-art techniques for the investigation of free extractable lipids in various materials are explained, which can be applied even in laboratory environments with limited technical equipment. The protocols cover sample preparation, extraction,	

	purification, analysis, as well as a brief overview of the data evaluation using lipid molecular proxies and compound-specific isotopes.
Keywords (separated by '-')	Alkanes - Biomarkers - Fatty acids - Gas chromatography - Lipid extraction - Lipid fraction - Molecular proxies - Preparative separation - Solid-phase extraction

Analysis of Lipids and Polycyclic Aromatic Hydrocarbons as Indicators of Past and Present (Micro)Biological Activity

Guido L.B. Wiesenberg and Martina I. Gocke

Abstract

Analysis of lipids and hydrocarbons was performed frequently in recent and ancient plant tissues, soils, sediments, peat deposits, oil, rocks, anthropogenic artifacts (archeological samples), and other materials to trace the contribution of different biological and anthropogenic sources of organic matter as well as environmental changes and the fate of organic matter like degradation. The approaches for the analysis of lipids and hydrocarbons strongly vary from traditional methodologies like thin-layer chromatography to universal approaches like pyrolysis, whereas the preparative separation of lipid fractions based on their polarity enables gas-chromatographic analyses of single fractions and compound-specific analysis of stable ($^{1}H/^{2}H$, $^{12}C/^{13}C$) and radioactive (^{14}C) isotope compositions. Often, lipid extraction operationally defines a subfraction of total lipids. On the one hand, free extractable lipids are obtained by extraction with organic solvents, whereas on the other hand, total samples or extraction residues are extracted for more polar lipid fractions using highly polar organic solvents and water, to release bound lipids. Also, procedures for extraction of free extractable lipids are diverse and mainly defined by the target of research and availability of instrumentation. In the current protocol, state-of-the-art techniques for the investigation of free extractable lipids in various materials are explained, which can be applied even in laboratory environments with limited technical equipment. The protocols cover sample preparation, extraction, purification, analysis, as well as a brief overview of the data evaluation using lipid molecular proxies and compound-specific isotopes.

Keywords: Alkanes, Biomarkers, Fatty acids, Gas chromatography, Lipid extraction, Lipid fraction, Molecular proxies, Preparative separation, Solid-phase extraction

1 Introduction

Lipids and polycyclic aromatic hydrocarbons (PAHs) comprise a broad diversity of biomarkers that have been analyzed with various techniques since almost 180 years [1–3]. Frequently, lipids and hydrocarbons were applied in various environmental settings and high diversity of materials and enabled assessment of vegetation change [4, 5], discrimination of vegetation types [6–8], environmental and climate changes [9, 10], contribution of various sources of microbial biomass [11–13], as well as tracing of burning residues

T.J. McGenity et al. (eds.), *Hydrocarbon and Lipid Microbiology Protocols*, Springer Protocols Handbooks,
DOI 10.1007/8623_2015_157, © Springer-Verlag Berlin Heidelberg 2015

[14, 15] and oil migration from source rocks to reservoirs [16, 17]. By compound-specific isotope analysis of lipids and PAHs, age determination became possible at a molecular level, and knowledge on turnover rates [18, 19] and insights into hydrology [20] were gathered. The applied techniques vary from extraction of lipidic compounds of samples like soils, sediments, rocks, plants, aerosols, and many other environmental sample types [21–24], to pyrolysis of undisturbed samples [25, 26]. While pyrolysis is a fast technique, extraction and analysis of lipid extracts gives the opportunity to analyze intact lipids rather than a mixture of intact lipids together with breakdown products of higher molecular weight compounds. The choice of solvents used for extraction defines the subfraction of lipidic compounds and PAHs that are extracted. While free extractable lipids are typically extracted with solvents or solvent mixtures of low to intermediate polarity depending on target substances [27–29], lipids bound to polar headgroups can be recovered with highly polar solvents including water-based solutions as, e.g., in the classical Bligh and Dyer method [30]. The current protocol aims at the investigation of free extractable lipids (Fig. 1) but not highly polar ones. So far, especially extractable lipids have been described to have high potential for preservation in geologically relevant timescales, thus providing environmentally significant biomarkers [31] in the long term. After extraction, lipid extracts can be either analyzed by gas chromatography after derivatization [32], or extracts are split into subfractions to avoid co-elution of substances during gas-chromatographic analyses [27] and further to enable proper compound-specific isotope investigations [19]. The aim of the current protocol was to provide a lipid extraction and separation procedure, which enables investigation of lipid fractions with low interferences and high reproducibility at comparatively low cost for instrumentation and consumables, enabling not only quantification and qualitative assessment via gas-chromatographic analysis but also compound-specific isotope analysis. The separation of lipid fractions is based on a combined method including fatty acid separation following the separation concept described by McCarthy and Duthie [33] and the separation of hydrocarbon fractions published by Radke et al. [34] including simplification and improvements in individual steps of the procedure. Several of the individual extraction and separation steps (Fig. 1) can be easily modified according to the individual laboratory prerequisites and target compound classes. Further, the current protocol gives hints to the evaluation of the gathered data by application of various molecular proxies dedicated to different purposes of data interpretation.

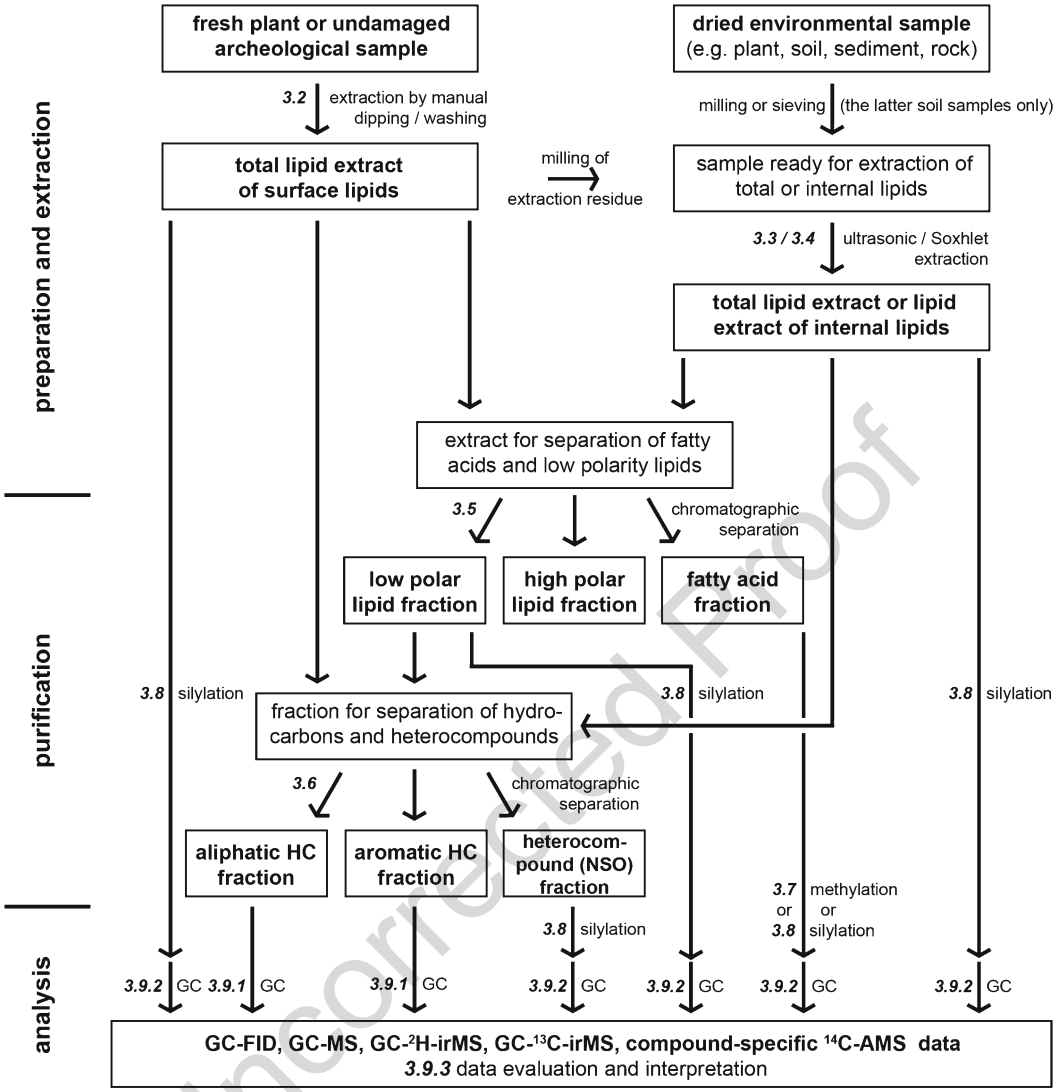


Fig. 1 Schematic summary of sample preparation, extraction, purification, and gas-chromatographic analysis of free extractable lipids described in the current protocol. *Bold terms* within *boxes* mark the most important parts, i.e., initial samples, extracts, and fractions that can be subjected to GC measurement and GC analysis itself. *Small numbers* written in *bold* and *italic letters* refer to the sections where the respective step is described in detail. *HC* hydrocarbon, *NSO* heterofunctionalized organic compounds containing nitrogen, oxygen, and sulfur

2 Materials

In order to enable contamination-free analysis of lipids and hydrocarbons, sample preparation and preparation of glassware need to be emphasized. To avoid contamination of plasticizers, it is generally recommended to use pre-cleaned or new glassware, the latter

especially relevant for pasteur pipettes, sample and fraction vials, caps, septa, filters, and glass wool. When using other tools like pipettors or pipette bulbs, it is recommended to check that the tools which might be in contact with samples, solvent, or solvent vapor do not cause any contamination. For example, use pipette bulbs made from silicon instead of rubber.

2.1 Preparation of Glassware, Glass Fiber Filters, Glass Wool, and Samples

Depending on the availability of equipment, several options are given in Sect. 3:

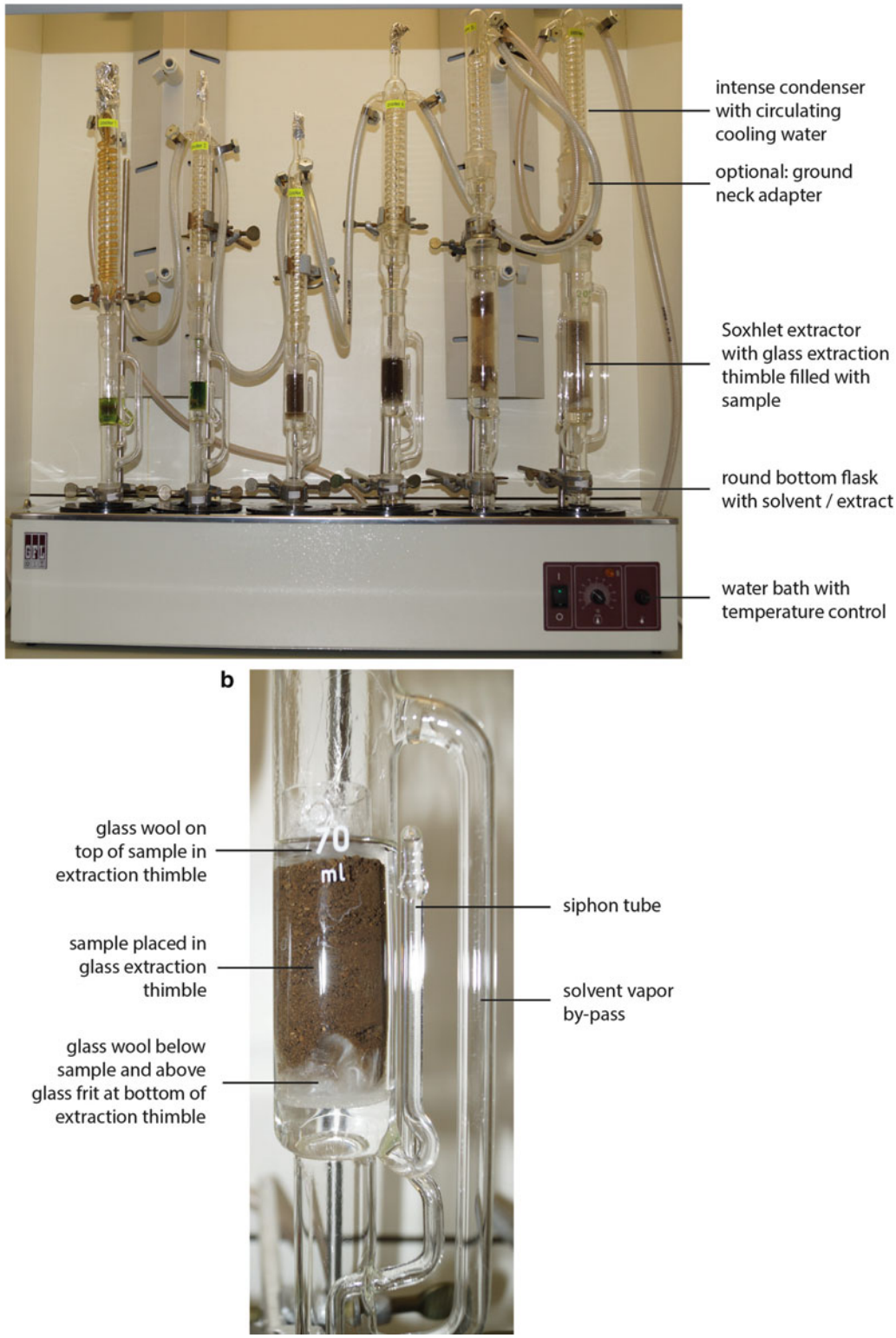
1. Dishwasher and dryer or facilities to clean glassware and drying oven dedicated to glassware.
2. Muffle furnace for heating of glassware, stainless steel connectors, and glass fiber filters or alternatively per analysis (p.a.) grade chemical dichloromethane, methanol, *n*-hexane for precleaning, depending on the solvent used in the individual steps of extraction and separation.
3. Samples must be free of any potential contamination, i.e., should not be touched without gloves. Previous contact of samples with, e.g., fossil fuel-derived liquids like drilling fluids or dust must be avoided during sampling and sample storage. Further, samples should be free of any marker, rubber, and plastic remains. If possible, plastics should be avoided during any step of sample storage to avoid contamination by plasticizers.[1] Ideally, samples and any physical or chemical fraction of samples should be stored dried in closed glass containers, glass vials, or aluminum foil under cool and dry conditions without exposition to sunlight. Details are referred to in the following sections.

2.2 Extraction of Surface Lipids and Hydrocarbons

Several extraction procedures are available, and depending on sample availability, focus of investigation, and equipment, the selection of sample preparation and choice of extraction procedure should be performed. In the following, protocols for extraction of plant cuticular waxes or extraction of lipids from surfaces [35, 36] of archeological artifacts (Sect. 2.2), plant-internal and total plant lipids, as well as lipids from soils, sediments, and rocks (Sects. 2.3 and 2.4) are explained [22, 27] (cf. Fig. 1). For rapid investigation of low sample amounts, ultrasonic extraction can be used (Sect. 2.3), whereas Soxhlet extraction is a standard technique (Sect. 2.4, Fig. 2) not only in biology, biogeochemistry, and geochemistry but also in other fields of science like food chemistry.

For extraction of cuticular lipids from leaf surfaces [35, 36] and from archeological artifacts:

1. Plant samples should be stored cool (<4°C) and analyzed shortly (1 h) after sampling in order to avoid erroneous results due to starving and degradation of biomass. If storage and



transport are required, samples should be kept frozen (< −18°C) in darkness and thawed directly before extraction. Archeological artifacts can be kept at room temperature. In general, extraction of surfaces should be performed on untouched, uncontaminated, and undamaged samples, without further sample pretreatment like drying or milling.

2. For plant and archeological samples: One round-bottom flask or beaker per sample (size of 50, 100, or 250 mL), depending on sample amount or size (*see* **Note 1**).
3. Solvent volume of dichloromethane or chloroform (e.g., VWR SupraSolv grade for GC 1.06054.2500 or 1.02432.2500) depends on sample size and surfaces to be extracted. Thus, the flask or beaker size should be selected, and an amount of solvent fitting into the respective round-bottom flask or beaker must be available. To improve extraction efficiency, 1–3% methanol (e.g., VWR SupraSolv grade for GC 1.00837.2500) can be added to dichloromethane or chloroform.
4. Metal stand and clamp for glass columns or vacuum manifold (e.g., Macherey-Nagel 730151) with long stainless steel (Macherey-Nagel 730106) or PTFE connectors (Macherey-Nagel 730564).
5. Per sample one 6 mL glass column (Macherey-Nagel 730172) and corresponding glass fiber filter (Macherey-Nagel 730192) (*see* **Note 2**).
6. 1–2 g sodium sulfate (Sigma-Aldrich 238597) per sample.
7. One pre-weighed glass vial (4–15 mL size) per sample for the extract with solvent-resistant septum and cap.
8. N_2 drying stand.

2.3 Ultrasonic Extraction of Low Sample Quantities

The ultrasonic extraction is ideal for low quantities of available samples, i.e., <2 g of samples of high density like soil, sediment, or rock and <0.1 g of plant tissues [10]. For low sample amounts, quantitative evaluation of the data might be less important than the qualitative information. For example, samples investigated for cuticular waxes (*see* Sect. 2.2) can be subjected to ultrasonic extraction.

1. Ca. 10 mL mixture of dichloromethane or chloroform (e.g., VWR SupraSolv grade for GC 1.06054.2500 or

Fig. 2 (Continued) Overview of the Soxhlet extraction setup. (**a**) Six samples of three different sizes positioned in an in-row water bath from the *left* to the *right*: two plant samples placed in a 30 mL extractor, two soil samples with high organic carbon concentrations (>2 mg g^{-1}) arranged in a 70 mL extractor, and two sediment samples with low organic carbon concentrations (<2 mg g^{-1}) weighed into a 200 mL extractor. (**b**) Detailed picture of a Soxhlet extractor equipped with a glass extraction thimble filled with a sieved (<2 mm) soil sample and glass wool plugs added below and on top of the soil sample

1.02432.2500) with methanol (e.g., VWR SupraSolv grade for GC 1.00837.2500) 93:7 (v/v) per sample.

2. Metal stand and clamp for columns or vacuum manifold (e.g., Macherey-Nagel 730151) with long stainless steel (Macherey-Nagel 730106) or PTFE connectors (Macherey-Nagel 730564).
3. Per sample one 6 mL glass column (Macherey-Nagel 730172) and corresponding glass fiber filter (Macherey-Nagel 730192) or similar system (cf. Sect. 2.2).
4. One pre-weighed glass vial (4–15 mL size) per sample for the lipid extract with solvent-resistant septum and cap.
5. N_2 drying stand.

2.4 Soxhlet Extraction

The classical Soxhlet extraction can be applied to any sample type. It is characterized by high reproducibility and is thus still a standard technique in geochemistry and food chemistry [37]. However, e.g., accelerated solvent extraction (ASE) can be used instead [27, 38] as extraction efficiency and reproducibility are similar or slightly higher compared to Soxhlet extraction. But as this technique is not widely distributed and especially the equipment is rather costly, the classical Soxhlet procedure is described in the following. The main limiting factors related to Soxhlet extraction are the number of extraction stands and duration of extraction. The number of individual extraction stands can be easily adjusted by applying large water baths and placing numerous metal stands with Soxhlet extractors surrounding the water bath. Using the correct extractor sizes, i.e., small extractors for small sample sizes, reduces the amount of solvent, shortens the time per extraction cycle, and thus increases sample throughput (Fig. 2). As at least two extraction cycles per sample are recommended for ASE, total extraction time can be similar or even longer compared with Soxhlet extraction, if 12 extraction stands are available for the latter. Further, if reusable glass Soxhlet extraction thimbles are used, costs per extraction can be significantly lower compared to ASE. In the following, Soxhlet extraction is described for three different sample sizes.

1. Soxhlet apparatus with cooler and water bath. If large water baths are available, several metal stands can be placed, and multiple Soxhlet extractions can be performed even at limited space compared to single individual Soxhlet extraction stands using, e.g., heating mantles. Soxhlet extractors and round-bottom flasks should be adjusted to sample sizes to be analyzed: For any plant sample and other samples of a maximum weight of 15 g material, 30 mL extractors and 50 mL round-bottom flasks can be used. For samples with comparatively low organic carbon content (0.1–10 mg g^{-1}) and high sample availability, 70 mL extractors and 100 mL round-bottom flasks

are suggested. For compound-specific ^{14}C analyses, extractors of 200 or 250 mL size and 250 mL round-bottom flasks can be used. If required, even larger setups can be chosen but will not be described here.

2. Reusable glass extraction thimble or single-use glass fiber extraction thimble in the respective size of the chosen extractor size (*see* **Note 3**, Fig. 2).
3. In total, 70, 120, or 250 mL mixture of dichloromethane or chloroform (e.g., VWR SupraSolv grade for GC 1.06054.2500 or 1.02432.2500) with methanol (e.g., VWR SupraSolv grade for GC 1.00837.2500) 93:7 (v/v) per sample, depending on the size of extractor (i.e., 70 mL solvent for 30 mL extractor, 120 mL solvent for 70 mL extractor, and 250 mL for 200 or 250 mL extractor).
4. Metal stand and clamp for columns or vacuum manifold (e.g., Macherey-Nagel 730151) with long stainless steel (Macherey-Nagel 730106) or PTFE connectors (Macherey-Nagel 730564).
5. One 3 or 6 mL glass column (Macherey-Nagel 730171 or 730172) and corresponding glass fiber filter (Macherey-Nagel 730191 or 730192) per sample or similar system (cf. Sect. 2.2).
6. One pre-weighed glass vial (4–15 mL size) per extract with solvent-resistant septum and cap.
7. N_2 drying stand.

2.5 Separation of Lipid Extracts into Fatty Acid and Low-Polarity Lipid Fractions

The principle of the method is based on the procedure described by McCarthy and Duthie [33]. The preparative separation into fatty acid and other lipid fractions is relevant in many environmental matrices like investigation of plant, soil, sediment, and rock samples [19, 39]. KOH-coated silica gel can be produced as described in the previously published method [33]. Alternatively, it can be produced and purchased on demand for a reasonable price at companies like Macherey-Nagel (www.mn-net.com).

1. Vacuum manifold (e.g., Macherey-Nagel 730151) with PTFE stop cocks (e.g., GL Sciences Inc, Japan 5010-60010) and long stainless steel (Macherey-Nagel 730106) or PTFE connectors (Macherey-Nagel 730564).
2. Per sample one 3 or 6 mL glass column (Macherey-Nagel 730171 or 730172) and corresponding glass fiber filter (Macherey-Nagel 730191 or 730192) or similar system.
3. 1.5–2.0 g KOH-coated (5%) silica gel 60 (0.063–0.2 mm) (Macherey-Nagel 815335.1) per sample. Note that if own KOH-coated silica gel is produced, the KOH coating could differ from the given value which influences the separation efficiency and should be tested.

4. Per sample two round-bottom flasks (50 mL).
5. Per sample ca. 50 mL dichloromethane (e.g., VWR SupraSolv grade for GC 1.06054.2500).
6. Per sample ca. 20 mL solvent mixture of dichloromethane (e.g., VWR SupraSolv grade for GC 1.06054.2500) and formic acid (e.g., VWR High Purity 97064-708) 99:1 (v/v).
7. If also highly polar and high molecular weight compounds are targeted, per sample 8 mL of a mixture of dichloromethane (e.g., VWR SupraSolv grade for GC 1.06054.2500) and methanol (e.g., VWR SupraSolv grade for GC 1.00837.2500) 1:1 (v/v) or higher proportions of methanol can be used. Note that during elution with methanol, the KOH is released from the silica gel and eluted with the lipid fraction.
8. 2–3 pre-weighed glass vials (4–15 mL size) for lipid fractions with solvent-resistant septum and cap per sample.
9. N_2 drying stand.

2.6 Separation of Total Lipid Extracts or of Low-Polarity Fractions into Aliphatic and Aromatic Hydrocarbons and Heterofunctionalized Organic Compounds

The separation is dedicated to separate mixtures of lipidic compounds into aliphatic and aromatic hydrocarbons as well as heterofunctionalized organic compounds containing nitrogen, oxygen, and sulfur (NSO compounds) [34]. This and similar methods are widely applied in biochemical and geochemical studies to preparatively separate low-polar lipid fractions to enable investigations of polycyclic aromatic hydrocarbons and of a variety of lipids that naturally occur only in trace amounts (e.g., steranes, hopanes) [13] and furthermore to facilitate compound-specific isotope determinations of the respective lipid fractions obtained [19, 20].

1. One glass pasteur pipette per sample for chromatographic separation
2. Pre-extracted glass wool
3. Ca. 1 g activated silica gel 100 Å per sample (*see* **Note 4**)
4. Per sample one clear and one amber 5–10 mL round-bottom or conical flask each
5. One clear and one amber autosampler vial for GC analysis, respectively, and one larger vial (4–15 mL)
6. Ca. 10 mL *n*-hexane (e.g., VWR SupraSolv grade for GC 1.00795. 2500) per sample
7. Ca. 4 mL mixture of *n*-hexane (e.g., VWR SupraSolv grade for GC 1.00795. 2500) and dichloromethane (e.g., VWR SupraSolv grade for GC 1.06054.2500) 1:1 (v/v) per sample
8. Ca. 4 mL mixture of dichloromethane (e.g., VWR SupraSolv grade for GC 1.06054.2500) and methanol (e.g., VWR SupraSolv grade for GC 1.00837.2500) 93:7 (v/v) per sample
9. N_2 drying stand

2.7 Methylation of Fatty Acids Using Boron Trifluoride/Methanol

Several methods are available for derivatization of fatty acids [40]. Silylation [41] and methylation [42] are frequently applied and lead to similar derivatization efficiency. However, methylated compounds are characterized by higher stability than silylated compounds, thus enabling longer storage of fractions. Therefore, methylation with boron trifluoride/methanol is recommended for fatty acids and thus described in the following for fatty acid derivatization, but silylation as explained in Sect. 2.8 would be an alternative method.

If not added in any previous step, an internal quantification standard is recommended to add before derivatization (*see* **Note 5**):

1. Up to 2 mg of fatty acids can be methylated. If more fatty acids are available, an aliquot should be separated.
2. Reacti-vial™ (ThermoScientific TS-13221) or glass vial (4–8 mL of size recommended) that can be closed airtight with cap and solvent-resistant septum.
3. 500 µL boron trifluoride/methanol (10%) (FLUKA 15716) per sample. Note that the reagent should be stored in a fridge (<4°C) and consumed within a few weeks after opening. Otherwise, the stability of the reagent cannot be guaranteed and might result in artifacts during methylation.
4. Ca. 2–3 mL dichloromethane (e.g., VWR SupraSolv grade for GC 1.06054.2500) per sample.
5. Ca. 1 g sodium sulfate (Sigma-Aldrich 238597) per sample.
6. 500 µL water of millipore quality per sample.
7. Glass pasteur pipette for filtration.
8. Pre-extracted glass wool.
9. One GC autosampler vial per sample, potentially with micro insert.
10. N_2 drying stand.

2.8 Silylation of Total Lipid Extracts, Fatty Acid, Alcohol, and Heterocompound Fractions

The silylation is a widely used procedure [40, 41], whereas several different reagents are used. To simplify, only derivatization with *N*, *O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) is explained in the following as the described method is simple to apply and universal for the mentioned fractions. However, shortcomings of the derivatization method are significantly stronger isotope correction that is required for compound-specific δ^2H and $\delta^{13}C$ analysis (*see* **Note 5**) and the limited stability of the derivatives. Due to the shortcomings, it is recommended to derivatize an aliquot instead of the whole sample or fraction. Aliquots of 1–2 mg substance of the mentioned fractions or total lipid extracts should be transferred to a GC autosampler vial. If required, deuterated standards such as

$D_{39}C_{20}$ acid (eicosanoic acid, Cambridge Isotope Laboratories Inc. DLM-1233-1), $D_{27}C_{18}$ alcohol (octadecanol, Cambridge Isotope Laboratories Inc. DLM-795-1), or other adequate internal standards can be added before silylation. As described for the methylation (cf. Sect. 2.7), a correction factor has to be determined for the carbon atoms as a consequence of the derivatization.

1. 50–100 µL *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (SUPELCO 33 027) for 1–2 mg extract or lipid fraction to be silylated
2. Per sample ca. 1 mL dichloromethane or chloroform (e.g., VWR SupraSolv grade for GC 1.06054.2500 or 1.02432.2500) for fatty acid, alcohol, and heterofunctionalized organic compounds or mixture of dichloromethane or chloroform with methanol (e.g., VWR SupraSolv grade for GC 1.00837.2500) 93:7 (v/v) for total lipid extracts
3. N_2 drying stand

2.9 GC Analysis and Data Evaluation

The gas-chromatographic analysis of samples including quantification and qualitative assessment as well as compound-specific isotope (δ^2H, $\delta^{13}C$, ^{14}C) determination strongly depends on research and analytical targets as well as sample types [19, 22, 28, 32, 38]. Therefore, in the following section, only a rough overview is given, and gas-chromatographic conditions as well as molecular ratios that are indicative for paleoenvironmental reconstruction or other processes calculated from lipid composition will be described exemplarily (e.g., [31]). For quantification and qualitative assessment, one major prerequisite is to choose adequate standard (series) (*see* **Note 6**). Compound identification can be also supported by using mass spectral libraries and comparing mass spectra as well as retention order of compounds to the literature.

1. GC equipped with FID and/or MS. The method descriptions are explained for a GC equipped with autosampler, split/splitless injector, He as carrier gas, and N_2 as makeup gas, so that some adaptations might be required, if other gases or injector types are installed. We use Agilent 6890 and 7890 GC equipped with FID or Agilent 5973 MSD with the same chromatographic setups and methods irrespective of the detector.
2. Packed liner (Agilent 5062-3587).
3. Deactivated FS pre-column of 5 m length and 0.32 mm diameter (Agilent 160-2325-5).
4. DB-5MS GC column of 50 m length, 0.2 mm diameter, and 0.33 µm film thickness (Agilent 128-5552).
5. All gases like N_2, He, H_2, and air should be of purity 4.0 or higher.

6. Optional for GC–MS measurements: Full ion scanning of m/z 50–550 is recommended for routine analysis. For high molecular weight compounds such as wax esters, the scan range can be extended to the maximum of the MS detector (e.g., m/z 850). Selected ion monitoring (SIM) of specific mass fragments is determined by the target compounds and related to their respective mass spectra and is not further discussed here.
7. Optional for compound-specific δ^2H and $\delta^{13}C$ measurements via GC-irMS: Purity of gases should be 5.0 or higher, and reference gases with known δ^2H and $\delta^{13}C$ values must be available. Correction for modifications like addition or exchange of H and C atoms has to be done (cf. Sect. 2.7).
8. Optional for compound-specific ^{14}C measurements via AMS: Proper checkup of potential contamination by fossil fuel or modern carbon is required for every analytical step. In addition, the checkup of the whole laboratory environment for ^{14}C contamination is required before starting compound-specific ^{14}C measurements via checking the historical use of the laboratories, and in the case that ^{14}C tracer analysis can be ruled out in the past, the so-called swipe tests have to be performed in collaboration with the AMS laboratories. Preparative GC is required to separate individual substances from one lipid fraction. The amount of individual lipids to be measured by AMS varies between facilities but should be in the range of 20–100 μg C, which determines the quantity of sample to be extracted and separated.

3 Methods

3.1 Preparation of Glassware, Glass Fiber Filters, Glass Wool, and Samples

All glassware should be pre-cleaned in order to avoid contamination and cross-contamination.

1. Cleaning of laboratory glassware: Clean by dishwasher or by hand using detergent for laboratory purposes, and ensure residue-free rinsing with deionized water followed by drying.
2. Depending on available facilities, heating of rinsed and new glassware in a muffle furnace at 500–550°C is recommended, followed by covering with aluminum foil until use. Note that glassware made from soda glass (e.g., pasteur pipettes) might be heated at lower temperatures as deformation might occur at high temperature. Alternatively, glassware should be cleaned with the solvents used in the respective analytical steps directly before use.
3. Consumables like glass wool and glass fiber filters can also be heated before use, cleaned with solvents as explained above, or pre-extracted in an ultrasonic bath or by Soxhlet.

3.2 Extraction of Surface Lipids and Hydrocarbons

The section is dedicated to the extraction of lipids from surfaces of plant and archeological samples by manual dipping of the sample in solvent or extracting surface lipids by flushing surfaces with solvents (Fig. 1) [35, 36].

1. After weighing sample or determining the surface area, place the available intact, undamaged archeological sample in a beaker or plant tissues in a round-bottom flask using tweezers.
2. Add dichloromethane or chloroform (GC grade) until sample is completely covered with solvent. Adding 1–3% methanol (GC grade) could improve extraction efficiency, especially if not only hydrocarbons but also fatty acids, alcohols, and other compounds of low and intermediate polarity are targeted. If only parts of an archeological artifact should be analyzed, e.g., if parts are contaminated and others are not, solvent can be flushed over the targeted area repeatedly and collected in a beaker.
3. Gently shake beaker or flask and make sure that major parts of the sample surface do not stick to the glass surface.
4. After 60 s, remove sample from solvent using tweezers.
5. Rinse sample while removing from beaker or round-bottom flask by flushing sample with dichloromethane or chloroform (GC grade) using a pasteur pipette or glass syringe, and make sure that added solvent is combined with the solvent in the beaker or round-bottom flask.
6. If extraction is performed in a beaker, transfer solvent to a round-bottom flask for volume reduction. During this step, it is recommended to rinse the beaker at least three times with fresh solvent and to make sure that this solvent is combined quantitatively with the already transferred solvent.
7. Let the solvent evaporate in fume hood until approximately 1–2 mL are left in the round-bottom flask or if rotary evaporator or similar equipment is available; evaporate using the underpressure required for the respective solvent until the desired solvent volume.
8. Install the 6 mL glass column with glass fiber filter using metal stand or vacuum manifold with connectors, and add 1–2 g sodium sulfate to each column for dehydration of the extract. Pre-clean the setup twice with the chosen solvent and discard the eluted solvent. Place extract vial underneath the column.
9. Transfer the extract to the 6 mL glass column using a pasteur pipette or glass syringe. If required, underpressure can be applied using the vacuum manifold.
10. Add another 1 mL of solvent to round-bottom flask and shake it gently.

11. Return to step 9 and repeat this cleaning procedure at least five times or until solvent in round-bottom flask is colorless for at least two rinsing steps. Be careful that the quantity of the solvent used for cleaning does not exceed the size of the sample vial.
12. After complete collection of lipid extract in the extract vial, remove solvent under gentle stream of N_2 or use a concentrator (e.g., Eppendorf).
13. Depending on the wetness of initial sample, the collected sample might contain water remains, thus requiring another drying step via a new syringe filled with sodium sulfate (steps 8–11).
14. Determine the dry weight of the pre-weighed sample vial. The net sample weight should be named "total cuticular lipid content" or "content of surface waxes" and normalized on mass of sample or surface area of investigation determined in step 1 in Sect. 3.2. The extract would be available for GC–MS analyses after derivatization (cf. Sects. 3.8 and 3.9), but preparative separation of lipid fractions (cf. Sect. 3.5ff) is recommended to ensure co-elution-free investigation of lipid composition during GC analysis. AU2
15. Dried lipid extracts can be stored in a dry, dark place at room temperature or fridge for months or years.

3.3 Ultrasonic Extraction of Low Sample Quantities

Low sample quantities can be subjected to ultrasonic extraction (Fig. 1) [10], which is also an option for fast screening of large sample numbers. More reliable results are obtained especially for larger sample amounts ($\gg$1 g) by Soxhlet extraction (*see* Sect. 3.4).

1. Samples must be dried (air- or oven-dried at $\leq$60°C or freeze-dried), milled to fine powder, or at least sieved to material <2 mm (for soil samples).
2. Weigh sample (<2 g of samples with high density like soil, sediment, or rock samples and <0.1 g of plant tissues) in a lockable glass vial fitting into the centrifuge, and note net sample weight.
3. Add 2 mL of solvent mixture dichloromethane or chloroform (GC grade) with methanol (GC grade) (93:7, v:v) to sample in the glass vial, close the vial and shake it.
4. Place the vial with the sample and solvent for 10 min in an ultrasonic bath or use vortex mixer for 30 s to enable proper mixing of the sample material with the solvent. To improve extractability, the temperature of the ultrasonic bath can be raised to 60°C.
5. Centrifuge samples at 800 *g* for 2 min.
6. Install the 6 mL glass column equipped with a glass fiber filter on a metal stand or vacuum manifold with connectors.

Pre-clean the setup twice with dichloromethane or chloroform (GC grade) and discard the eluted solvent. Place a pre-weighed vial for the lipid extract underneath the outlet of the connector.

7. Transfer the supernatant solvent of the extraction vial to the 6 mL glass column.
8. Return to step 3 and repeat steps 3–7 (except for step 6) at least five times, and combine the extract in one glass column and sample vial, respectively. If required, underpressure applied to vacuum manifold can increase the filtration speed.
9. Remove eluted solvent from the sample vial using a gentle stream of N_2 or a concentrator.
10. Determine the dry weight of the lipid extract in the pre-weighed sample vial. The net sample weight should be named "total extractable lipid content" or "content of internal lipids" and normalized on mass of sample or content of organic carbon. The lipid extract would be available for GC–MS analyses after derivatization (cf. Sects. 3.8 and 3.9), but preparative separation of lipid fractions is recommended to ensure co-elution-free investigation of lipid composition (cf. Sect. 3.5ff).
11. Dried lipid extracts can be stored in a dry, dark place at room temperature or fridge for months or years.

3.4 Soxhlet Extraction

The classical procedure for lipid extraction of a variety of biological, geological, and environmental samples is Soxhlet extraction (Figs. 1 and 2) [37], which is the method of choice for quantitative extraction of any sample type of higher quantity ($\gg$1 g).

1. Make sure that contamination-free, dried, and milled material of plant tissues, rock, soil, or other environmental samples is available. Soil samples could be also sieved (<2 mm). If sample material is available as very finely grained powder or the material is very rich in organic matter (e.g., peat or coal), also preheated quartz sand can be mixed with the sample to ensure proper penetration by solvent during extraction.
2. Choose size of the Soxhlet extractor depending on sample size and purpose of analysis.
3. Add plug of pre-extracted or combusted glass wool to extraction thimble (Fig. 2).
4. Fill weighed sample to extraction thimble, and cover it with another plug of glass wool.
5. Install Soxhlet extractor with the following setup: Place a round-bottom flask of the size corresponding to chosen extractor filled with the solvent mixture dichloromethane or chloroform (GC grade) with methanol (GC grade) (93:7, v:v) at the base of the setup in the water bath. Above, the extractor should be placed with the sample to be extracted in the extraction

thimble. On top, the cooler should be installed. Check all ground necks and potential glass adapters used to connect the glassware that no sample particles or glass wool inhibit proper tightness of glass connections between extractor and cooler, as well as between extractor and round-bottom flask.

6. Turn cooling water on and adjust temperature of the water bath to 54°C. Let the extraction run for at least 20 cycles. The number of cycles can be reached after approximately 24 h for 30 mL extractors, 36–48 h for 70 mL extractors, and 48–96 h for 200–250 mL extractors, respectively. The time span depends on evaporation and cooling of solvents as well as sample filling of extraction thimble, i.e., the lower the filling, the longer the duration of the extraction.
7. Check the extraction setup twice per day for tightness, sufficient amount of solvent in the circulation system, and proper circulation of the cooling liquid. If required, add further dichloromethane or chloroform (GC grade) with methanol (GC grade) (93:7, v:v) or adjust circulation of the cooling liquid and tightness.
8. After at least 20 extraction cycles, move the whole extraction setup out of water bath, and let it cool down to room temperature.
9. Disconnect Soxhlet extractor from cooler. Flush remaining solvent from Soxhlet extractor into round-bottom flask by gentle tilting of the whole setup. Make sure that solvent passes through the siphon and not the vapor bypass.
10. Dissemble the setup and rinse ground necks with the solvent mixture dichloromethane or chloroform (GC grade) with methanol (GC grade) (93:7, v:v) in the way that the solvent used for rinsing is transferred to the round-bottom flask. Discard the extraction residue after drying of solvent in the extraction thimble or keep it for extraction and pyrolytic analysis of bound lipids (not explained in this protocol).
11. Remove the solvent from the round-bottom flask by rotary evaporation or similar devices for solvent reduction.
12. Install a 3 or 6 mL glass column with a glass fiber filter using a metal stand or vacuum manifold with connectors. Pre-clean the setup twice with dichloromethane or chloroform (GC grade), and discard the eluted solvent. Place a pre-weighed extract glass vial underneath.
13. Transfer the lipid extract quantitatively from the round-bottom flask to the glass column, and rinse the round-bottom flask at least five times or until solvent remains colorless for at least two rinsing steps. The solvent should be transferred always to the same glass column.

14. Reduce the volume of the eluted solvent in the lipid extract vial until dryness using a gentle stream of N_2 or a concentrator.
15. Determine the dry weight of the pre-weighed extract vial. The net sample weight should be named "total extractable lipid content" or "total content of internal lipids," if plant-internal lipids have been extracted after removal of cuticular lipids. The extract would be available for GC–MS analysis after derivatization (cf. Sects. 3.8 and 3.9), but preparative separation of lipid fractions is recommended to ensure co-elution-free investigation of lipid composition (cf. Sect. 3.5ff).
16. Dried lipid extracts can be stored in a dry, dark place at room temperature or fridge for months or years.

3.5 Separation of Lipid Extracts into Fatty Acid and Low-Polarity Lipid Fractions

Lipid extracts need to be separated into fractions of different polarity to improve qualitative and quantitative evaluation of lipid data obtained during GC analysis. As fatty acids and high molecular weight and high-polarity compounds can be dominant in various environmental sample types, this method enables the preparative separation of fatty acids from low-polarity lipids and high molecular weight and polarity compounds (Fig. 1) [19, 33, 39]. The described method is optimized for ca. <30 mg lipid extract obtained from plant, sediment, rock, soil, or similar samples (*see* **Note 7**). Depending on the targeted substance classes and composition of the lipid extract, this separation step might be skipped, e.g., if only polycyclic aromatic hydrocarbons or aliphatic hydrocarbons should be analyzed and the content of high molecular weight and high-polarity compounds as well as fatty acids is low. It is recommended to test for a proper separation of different lipid fractions, if sample series of unknown composition should be separated.

1. The glass column equipped with a glass fiber filter is attached to the stop cock, which is placed on top of the PTFE or stainless steel connector, where the latter is inserted into the lid of the vacuum manifold.
2. Add ca. 1.5–2.0 g KOH-coated silica gel to the glass column.
3. Place beaker underneath the connector outlet.
4. Fill the column with dichloromethane (GC grade), and rinse silica gel with two column volumes of dichloromethane (GC grade). During rinsing, the stop cock might be turned to properly rinse its interior. Make sure that until the end of the separation, the silica gel is always covered with a bit of solvent and does not fall dry, which would decrease the separation potential of the silica gel.
5. Remove air bubbles from silica gel (*see* **Note 8**).

6. Discard eluted solvent from the rinsing step, and place first round-bottom flask underneath the connector outlet.
7. Dissolve the lipid extract in 0.5 mL dichloromethane (GC grade), agitate the solution by using a vortex mixer or ultrasonic bath, and potentially increase the temperature not exceeding 35°C to prevent boiling of the solvent.
8. Transfer the lipid extract with a pasteur pipette or a glass syringe to the silica gel column.
9. Repeat steps 7–8 at least five times or until solvent stays colorless for at least two rinsing steps.
10. Opening the stop cock might be already needed while repeating steps 7–8, which might require adding further dichloromethane (GC grade) to the silica gel column to prevent the silica gel from falling dry.
11. To elute the low-polarity fraction, add sequentially ca. 25 mL dichloromethane (GC grade) every time shortly before the silica gel falls dry. In the end, ca. 30 mL dichloromethane should have been passed into the round-bottom flask. During elution of the fraction, the stop cock should be kept always open to prevent loss of material within the stop cock.
12. Close stop cock and replace the first round-bottom flask with the low-polarity lipid fraction by an empty round-bottom flask that is dedicated to the fatty acid fraction. While removing the round-bottom flask, rinse the connector outlet and ground neck of the flask with a bit of solvent. While eluting the last 5–10 mL of the fraction, the color at the base of the silica gel column should be lighter compared to the top of the column. If this did not occur and the whole silica column remained colored, probably the separation is inappropriate, and some parts of the next fraction, i.e., fatty acids, have been already eluted. In this case, the eluted fraction should be reduced in volume and separated again over a new KOH-silica column. For similar samples, the amount of lipid extract to be placed on one KOH-silica column should be reduced. To check the lipid fractions for a proper separation, aliquots of the fractions can be checked via GC (cf. Sects. 3.8 and 3.9).
13. The elution of fatty acids starts with switching the solvent added to the column to the mixture dichloromethane (GC grade) formic acid (high purity) (99:1, v/v) and opening the stop cock again.
14. Sequentially elute ca. 20 mL of the dichloromethane (GC grade) formic acid (high purity) mixture in the round-bottom flask. During elution of the fraction, the stop cock should not be modified to prevent loss of material within the stop cock. Check during elution if color bands move downward within

the silica. When these color bands have completely moved downward, add another 3–5 mL to ensure complete elution of the fraction. If substances with several functional groups like di- or tricarboxylic acids are included in a lipid extract, which could be indicated by slowly moving color bands, this could require application of larger solvent volumes due to stronger interactions of the acids with the KOH of the silica gel.

15. After elution of the fatty acid fraction, the round-bottom flask can be removed. While removing the round-bottom flask, rinse the connector outlet and ground neck of the flask with a bit of solvent that should be flushed into the round-bottom flask with the fatty acid fraction.
16. Reduce solvent in the round-bottom flasks of low-polarity and fatty acid fractions by rotary evaporation or similar techniques to a volume of 1–2 mL.
17. Transfer the lipid fractions from both round-bottom flasks to pre-weighed vials labeled with "low-polarity compounds" (first fraction) and "fatty acids" (second fraction). Rinse round-bottom flasks with low amounts of dichloromethane (GC grade) for both fractions, and combine the solvent from rinsing steps in the respective fraction vial.
18. Fractions can be dried under a gentle N_2 stream. The weight might be determined, and then the closed vials can be stored in a dry, dark place at room temperature or fridge for months or years.
19. Optional step: To recover high-polarity and high molecular weight compounds from the silica column, ca. 4 mL mixture of dichloromethane (GC grade) and methanol (GC grade) 1:1 (v/v) can be used after placing a vial for the polar fraction underneath the connector outlet.
20. Let the column dry in the fume hood, and discard silica gel in special waste for silica.

3.6 Separation of Total Lipid Extracts or of Low-Polarity Fractions into Aliphatic and Aromatic Hydrocarbons and Heterofunctionalized Organic Compounds

The current method is optimized for soil, rock, sediment, and plant samples with comparatively low amounts of heterocompounds (i.e., compounds containing also N, S, and/or O like alcohols, ketones, and other compounds as well as fatty acids if lipid extracts are separated). It is dedicated to the preparative separation of aliphatic and aromatic hydrocarbons as well as other lipids, depending on the respective pretreatment (Fig. 1) [34]. To avoid overload of columns, the separation of lipid extracts into fatty acid and low-polarity lipid fractions (cf. Sects. 2.5 and 3.5) is recommended before splitting of hydrocarbons. Depending on the chemical composition of the extract or lipid fraction to be separated, the upper limit of samples to be separated varies between 10 mg (without

previous separation step) and 20 mg (after previous separation step).

1. Insert a small plug of glass wool into the glass pasteur pipette, and push it downward, e.g., by using the tip of another pasteur pipette.
2. Add a column of ca. 5.0–5.5 cm height of activated silica gel on top of the glass wool.
3. Fix the pasteur pipette, e.g., in a metal stand and place a beaker underneath.
4. Flush approximately 4–5 mL *n*-hexane (GC grade) through the column into the beaker for cleaning and conditioning purposes.
5. Remove air bubbles from silica (*see* **Note 8**)
6. Replace beaker by a clear round-bottom or conical flask to collect aliphatic hydrocarbons.
7. Dissolve sample in a small amount (ca. 200 μL) of *n*-hexane (GC grade), and improve dissolution by using a vortex mixer.
8. Transfer the sample to the column.
9. Repeat steps 6–7 five times to ensure a quantitative transfer of the sample.
10. Add sequentially another 4 mL of *n*-hexane (GC grade) to the column.
11. Replace the clear round-bottom or conical flask by an amber round-bottom or conical flask for collection of aromatic hydrocarbons.
12. Add sequentially 5 mL of the mixture *n*-hexane (GC grade): dichloromethane (GC grade) 1:1 (v/v) to the column.
13. Replace the amber round-bottom or conical flask by fraction vial for the collection of heterocompounds.
14. Add sequentially 4 mL of the mixture dichloromethane (GC grade):methanol (GC grade) 93:7 (v/v).
15. The solvent can be reduced under a gentle N_2 stream or by using a rotary evaporator or concentrator. Afterward, aliphatic hydrocarbons should be transferred with *n*-hexane (GC grade) to a clear autosampler vial and aromatic hydrocarbons with *n*-hexane (GC grade) to an amber autosampler vial.
16. The solvent of the fraction with heterocompounds can be removed until dryness, and this fraction can be stored in a dry and dark place for months or years. Aliphatic and aromatic hydrocarbons should not be dried completely and stored at <4°C. Also these fractions can be stored for months or years.
17. Let the solvent evaporate from the silica column in the fume hood, and discard the silica gel in special waste for silica.

3.7 Methylation of Fatty Acids Using Boron Trifluoride/Methanol

Reproducible derivatization techniques are required for quantitative investigations of polar lipid fractions such as fatty acids as well as lowest interferences for compound-specific isotope analysis [40]. For fatty acids, methylation has been proven to fulfill both criteria, which is why we recommend this method for fatty acid analysis [42].

1. Dissolve the total fatty acid fraction or an aliquot of total fatty acids (<2 mg fatty acids can be methylated with the described method) in 300 µL dichloromethane (GC grade) in fraction vial.
2. Optional: If required, add an internal standard to the fraction vial using a glass syringe.
3. Add 500 µL boron trifluoride/methanol to the fraction vial.
4. Close the vial and make sure that it is properly tightened. Place the vial in a heating block or drying cabinet at 60°C for 15 min. If required, leave 1–2 min more for equilibration of the temperature.
5. Remove the vial afterward from the heating block, and let it cool down to room temperature before opening the vial.
6. Add 500 µL water of millipore quality to the fraction vial, close the vial again, and use the vortex mixer to properly mix the liquids.
7. Centrifuge the vial for 1 min at 300 *g*.
8. Insert a small plug of glass wool into a glass pasteur pipette, and push it downward, e.g., by using the tip of another pasteur pipette.
9. Add 0.5–1 g sodium sulfate to the pasteur pipette, and clean it with ca. 1 mL dichloromethane (GC grade). Afterward, place an autosampler vial underneath the pasteur pipette to collect the methylated fatty acids.
10. Transfer the lower (organic) phase from derivatization vial to the filled glass pasteur pipette.
11. Add another 100 µL dichloromethane (GC grade) to the derivatization vial, use the vortex mixer, and repeat steps 7 and 10–11 at least five times or until the organic phase remains colorless at least three times to enable quantitative transfer of sample.
12. Add another 200–400 µL dichloromethane (GC grade) to the pasteur pipette for complete elution of fatty acids from sodium sulfate.
13. Afterward, the solvent volume in the autosampler vial can be reduced under a gentle N_2 stream, and the methylated fatty acids can be transferred to a micro insert, if required.

14. Methylated samples can be stored in a dry place in darkness at room temperature (<25°C) and in a fridge or frozen for months to years.

3.8 Silylation of Total Lipid Extracts, Fatty Acid, Alcohol, and Heterocompound Fractions

A more universal approach for the derivatization of a high diversity of lipid compounds is the silylation technique (Fig. 1) [40, 41], which is described in the following:

1. Transfer the total sample or an aliquot to a GC autosampler vial.
2. If the extract or fraction is dried, dissolve the sample in 100–200 μL, or otherwise adjust solvent to an amount of ca. 100–200 μL dichloromethane or chloroform (GC grade) for fatty acid, alcohol, and heterocompound fractions or a mixture of dichloromethane or chloroform (GC grade) with methanol (GC grade) (93:7, v/v) for total lipid extracts deriving from ultrasonic or Soxhlet extraction.
3. Add 50–100 μL N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) to the sample and close vial carefully.
4. Place the sample vial in the heating block or drying cabinet for 1 h at 80°C.
5. Remove the sample vial from the heating block, and let it cool down to room temperature before opening the vial.
6. Open the vial under a fume hood, and leave it open for ca. 15 min in fume hood to evaporate remains of the reactants. Note that the solvent should not completely evaporate as otherwise crystals form during evaporation, which commonly cannot be redissolved again, easily.
7. Add dichloromethane or chloroform (GC grade) to adjust the targeted solvent volume for GC analysis. If required, the silylated sample can be transferred to a micro insert for the GC autosampler vial.
8. The silylated total lipid extract or lipid fractions should be kept in a fridge (<8°C), and measurement within 24 h after derivatization is recommended. Silylated samples can be stored for 1 week, but depending on the sample, measurements can be impossible already after 48 h.

3.9 GC Analysis and Data Evaluation

The following section is dedicated to qualitative and quantitative as well as compound-specific isotope analysis of total lipid extracts and lipid fractions (Fig. 1) [19, 22, 28, 32, 38]. For simplification, only the chromatographic programs are explained, whereas specific requirements for mass spectrometric investigations of lipid fractions like selected ion monitoring (SIM) or details on compound-specific isotope determinations are not included. Also, only a selection of frequently applied molecular proxies for the assessment of different

vegetation types, degradation, and microbial vs. higher plant biomass or oil and source rock characterization are mentioned. For further details, respective textbooks (e.g., [31]) or research articles should be studied.

1. Analysis of aliphatic and aromatic hydrocarbon fractions:
   (a) If required, add an internal standard such as $D_{50}C_{24}$ *n*-alkane (tetracosane, Cambridge Isotope Laboratories Inc. DLM-2209-0.5), D_{10}-phenanthrene (Aldrich 364622), or other standard in the required amount. If necessary, aliphatic and aromatic hydrocarbon fractions can be measured without standard first and after standard addition, again.
   (b) If aliphatic and aromatic hydrocarbon fractions are dried, they should be dissolved in *n*-hexane (GC grade). Measurement is performed at a concentration of 10–20 μg/μL with 1 μL injected at 70°C in splitless mode.
   (c) Injector and detector temperature are both set to 320°C.
   (d) Constant injection temperature is kept for 2 min. Oven temperature is ramped at 10°C/min until 120°C and further at 5°C/min until 320°C. The final temperature is kept constant for 20 min.
2. Analysis of total lipid extracts, fatty acid, alcohol, and heterocompound fractions:
   (a) If total lipid extracts and lipid fractions are dried, they should be dissolved in dichloromethane or chloroform (GC grade) or a mixture of dichloromethane or chloroform (GC grade) with methanol (GC grade) (93:7, v/v). Measurement is performed at a concentration of 10–20 μg/μL with 1 μL injected at 50°C in splitless mode.
   (b) Injector and detector temperature are both set to 320°C.
   (c) Constant injection temperature is kept for 2 min. Oven temperature is ramped at 5°C/min until 120°C and further at 2°C/min until 210°C and 3°C/min until 320°C. The final temperature is kept constant for 20 min.
3. Interpretation of lipid composition based on lipid molecular proxies and compound-specific isotope values:
   (a) Chemotaxonomy

   Lipid molecular proxies have been frequently applied to distinguish between organic matter deriving from microorganism and higher plant biomass, as well as between different higher plant communities. This differentiation is possible due to the chemotaxonomic significance of various lipids [23, 43–46], although in recent years, doubts arose if this approach is reasonable. Critical analysis of the data and for samples like soils and sediments, a

proper cross-check with other data (e.g., pollen and other chemical properties than lipids can be chosen for sediments or land-use history for soils) is recommended. For alkyl lipids like alkanes, fatty acids, and alcohols, straight-chain homologues between approximately 12 carbons and maximum 34–37 carbons are detected. Compounds of comparatively longer chain length (alkanes $>C_{24}$ [47], fatty acids $>C_{19}$ [47], and alcohols $>C_{21}$ [3]) are typically enriched in higher plant biomass, whereas often the shorter-chain length homologues are enriched in organic matter produced by microorganisms [47]. Therefore, the **a**verage **c**hain **l**ength (ACL, Eq. 1) is used to estimate fresh higher plant input vs. degraded biomass and microbial remains in soil and sedimentary archives:

$$\mathrm{ACL} = \sum (z_n * n) / \sum z_n \qquad (1)$$

with n as the number of carbons within a compound and z_n as the quantity of the respective compound. Further, several ratios of long-chain alkyl lipids can be used to discriminate certain plant groups: Often grasses are characterized by a dominance of $n{-}C_{31}$ and/or $n{-}C_{33}$ alkanes, whereas $n{-}C_{27}$ is the dominant long-chain alkane in many woody plants [8]. However, this approach might be biased as also coniferous trees contain large amounts of $n{-}C_{31}$ and/or $n{-}C_{33}$ alkanes [48], thus questioning the general applicability of this approach and demanding further parameters like specific biomarkers or pollen to confirm the results. Relative portions of tree and grass biomass contributing to organic matter thus might be estimated using the long-chain **alk**ane ratio (AlkR, Eq. 2):

$$\mathrm{AlkR} = n - C_{27} / (n - C_{31} \text{ and/or } n - C_{33}) \qquad (2)$$

Within the fatty acids, a discrimination between plant communities of contrasting photosynthetic pathway can be accomplished using the **c**arboxylic **a**cid **r**atio (CAR; Eq. 3):

$$\mathrm{CAR} = n - C_{24} / (n - C_{22} + n - C_{26}) \qquad (3)$$

with CAR > 0.67 indicating C_4 vegetation and CAR < 0.67 suggesting C_3 vegetation as main source of organic matter [49]. As explained above, the sole use of this parameter to assess C_3 vs. C_4 vegetation-derived biomass should be avoided. Similarly, long-chain alcohols might be used to distinguish between C_3 and C_4 vegetation, with C_4 vegetation often enriched in very long-chain alcohol homologues and n-C_{32} occurring only in C_4

plants [46, 50]. Furthermore, various biomarkers including cyclic, branched, and unsaturated compounds exist within free extractable lipids for the identification of sources of organic matter in any kind of environmental setting as, e.g., for different communities of microorganisms [13, 31, 44, 51, 52].

(b) Oil and source rock characterization as well as assessment of degradation

Besides chemotaxonomic significance, various lipid parameters can be applied to assess degradation of organic material and to characterize, e.g., crude oils, oil generation, migration and basins, as well as potential source rocks. Fresh biomass of higher plants is characterized by odd-over-even dominance for alkanes and even-over-odd dominance for fatty acids and alcohols [3], whereas the respective counterparts originate from degradation. The **c**arbon **p**reference **i**ndex (CPI; Eqs. 4, 5, and 6) thus indicates if organic matter is fresh (high CPI) or to which degree it has been degraded (values close to 1):

$$\mathrm{CPI_{alk}} = \left[\left(\sum \mathrm{C_{25-33\ odd}}/\sum \mathrm{C_{24-32\ even}}\right) + \left(\sum \mathrm{C_{25-33\ odd}}/\sum \mathrm{C_{26-34\ even}}\right)\right]/2 \quad (4)$$

$$\mathrm{CPI_{fa}} = \left[\left(\sum \mathrm{C_{20-32\ even}}/\sum \mathrm{C_{19-31\ odd}}\right) + \left(\sum \mathrm{C_{20-32\ even}}/\sum \mathrm{C_{21-33\ odd}}\right)\right]/2 \quad (5)$$

$$\mathrm{CPI_{alc}} = \left[\left(\sum \mathrm{C_{22-32\ even}}/\sum \mathrm{C_{21-31\ odd}}\right) + \left(\sum \mathrm{C_{22-32\ even}}/\sum \mathrm{C_{23-33\ odd}}\right)\right]/2 \quad (6)$$

for alkanes ($\mathrm{CPI_{alk}}$), fatty acids ($\mathrm{CPI_{fa}}$), and alcohols ($\mathrm{CPI_{alc}}$), respectively. Depending on the application or research question, the CPI can be adjusted to other carbon numbers, like shorter-chain length. In analogy to the $\mathrm{CPI_{alk}}$, the **o**dd-over-**e**ven **p**redominance (OEP; Eq. 7) is a frequently used parameter with similar meaning:

$$\mathrm{OEP} = \sum \mathrm{C_{27-33\ odd}}/\sum \mathrm{C_{26-32\ even}} \quad (7)$$

The **r**atio of **s**aturated vs. **u**nsaturated $\mathrm{C_{16}}$ and $\mathrm{C_{18}}$ fatty acids (RSU, Eq. 8) is a further measure for the degree of preservation of organic matter, as both plants and microbial biomass are rich in unsaturated fatty acids [47], and the latter are susceptible to fast degradation.

$$\mathrm{RSU} = (\mathrm{C_{16:0}} + \mathrm{C_{18:0}})/(\mathrm{C_{16:1}} + \mathrm{C_{18:1}} + \mathrm{C_{18:2}}) \quad (8)$$

For source rock and oil characterization, numerous parameters are available, such as several ratios derived, e.g., from alkylated phenanthrenes (methyl phenanthrene

indices, MPI) or hopanes and homohopanes (C_{31}–C_{35}). Details for these proxies can be found elsewhere [31].

(c) Environmental parameters and (anthropogenic) pollution Another frequent application of lipid molecular proxies is to trace environmental changes and (anthropogenic) pollution. The ratio of the two isoprenoid alkanes pristane and phytane is a measure for redox conditions during deposition of a sediment [53], with values >1 indicating oxic conditions. As the relative portions of both compounds are susceptible to thermal degradation, further indices which include *n*-C_{17} and *n*-C_{18} alkanes (like *n*-C_{17}/pristane and *n*-C_{18}/phytane as well as the **i**soprenoid-**a**lkane **r**atio; IAR, Eq. 9) can be helpful to assess environmental conditions and the degree of biodegradation by aerobic bacteria.

$$\mathrm{IAR} = (\mathrm{Pr} + n - \mathrm{C}_{17})/(\mathrm{Ph} + n - \mathrm{C}_{18}) \quad (9)$$

Further biomarkers deriving from alkenones and glycerol dialkyl glycerol tetraethers are used to reconstruct (paleo) environmental temperature records, as their source organisms change numbers of double bonds, alkyl groups, and cyclic structures in their membranes as a result of temperature [10, 54].

Compound-specific δ^2H isotopic composition of various lipids is used to assess hydrological changes at the time of their biosynthesis [20, 55, 56]. Similarly, the compound-specific δ^{13}C isotopic composition allows for assessment of environmental influences like hydrological conditions, elevation, and exposition, on plant biosynthesis [57] but also for distinction of C_3- and C_4-plant-derived organic matter [19, 46, 58], as well as for detection of admixtures of fossil fuel [59]. Also fraction- and compound-specific radiocarbon dating has been used to identify pollution by fossil fuel [59, 60] as well as for age determination [60, 61].

Apart from assessing pollution by fossil fuel-derived carbon, e.g., via polycyclic aromatic hydrocarbons [14, 62, 63] (http://www.epa.gov/airtoxics/hlthef/polycycl.html), various biomarkers have been used to trace human activity, e.g., via sterols [64] and other compounds [65].

4 Notes

1. It must be ensured that samples fit into flask or beaker or that diameter of beakers is large enough to enable proper extraction of undisturbed surfaces of samples. That is, beakers or glass

bowls with a larger diameter could be required for large-sized samples.

2. Similar filter systems such as syringe filters can be used, but higher risk exists in terms of blocking of filters compared to the mentioned glass columns. Other filter systems like folded filters used in combination with funnels might entail insufficient elution of extracts.
3. In comparison to glass fiber or other Soxhlet extraction thimbles that are designed for single use, reusable glass extraction thimbles pay off after 5–10 uses.
4. Activation of silica gel should be performed by placing silica gel over night in a beaker covered with aluminum foil at 120°C in a drying cabinet or muffle furnace. Note that after removal of the silica gel from the drying cabinet or muffle furnace, the silica gel should be stored in a sealed glass flask, e.g., Erlenmeyer flask with ground neck. Either, the glass flask should be stored in a desiccator or activation should be performed, daily.
5. For the analysis of 1–2 mg fatty acids, commonly 5–10 μg standard are sufficient. For quantification of fatty acids by GC analysis or investigation of compound-specific $\delta^{13}C$ analysis, deuterated standards like $D_{39}C_{20}$ acid (eicosanoic acid, Cambridge Isotope Laboratories Inc. DLM-1233-1) are recommended. If compound-specific $\delta^{2}H$ analysis or compound-specific ^{14}C dating is planned as further analysis, specific branched or cyclic acids can be recommended as internal quantification standard, which are not included in the respective sample. For phospholipid fatty acid analysis, methyl nonadecanoate (e.g., Sigma N5377) is frequently used. However, this compound can be present in environmental samples, which might result in difficulties during quantification. This problem can be overcome at least partially by the application of external standards and their use for calibration as well as taking only aliquots of fatty acid fractions with internal standard for quantification and leaving other aliquots without any internal standard for compound-specific isotope analysis. Note that for compound-specific isotope analysis, external standards such as dodecanoic (lauric) acid (Aldrich W261408) can be measured via GCirmMS prior and after methylation to assess the correction factor for the added carbon of the methyl group during methylation. Alternatively, the carbon isotope composition of the boron trifluoride/methanol reagent can be measured by elemental analysis–isotope ratio mass spectrometry.
6. External standard series should be used for an assessment of the calibration curve and of response factors as well as for determination of retention times and thus identification of compounds. Further, it is recommended to add internal standards

to fractions that are measured via GC for quality control and quantification purposes. However, it must be ensured that the internal standards do not co-elute with other compounds or, if this is not possible, that lipid fractions are measured with and without a spiked standard. It has to be taken into account that internal standards are added in adequate quantity, i.e., in the average concentration of the target compounds and that they do not cause problems during further analytical phases, e.g., if deuterated standards are added (cf. Sect. 2.7).

7. The separation efficiency is influenced by the composition of the lipid extract. Large amounts of high molecular weight and high-polarity compounds as well as fatty acids may require the splitting of an aliquot of the lipid extract to allow for complete separation of the lipid fractions. If the lipid extract is dominated by low-polarity compounds, up to 70 mg of lipid extract can be separated by a single column.
8. Remove air bubbles from silica gel using one of the following optional techniques or a combination of these: Close stop cock and either carefully knock on the side of the column until air bubbles are removed. Take care that during this procedure, the column stays properly attached to the stop cock. Alternatively, a spatula or pasteur pipette might be used to carefully stir the silica gel, which also releases air bubbles. Make sure to add sufficient solvent to saturate the silica gel during the air removal. After these steps, another 3–4 mL of solvent might be eluted through the column to ensure a proper cleaning and conditioning of the column. As an alternative to both mentioned methods, also under- or overpressure might be used to enforce air bubbles moving downward through the column. A further approach is to suspend a larger amount of silica gel in dichloromethane (GC grade) first, and then flush the suspended silica gel into the column. After removal of air bubbles, the stop cock should be closed, and a small amount of solvent supernatant should be kept on top of the silica gel.

References

1. Liebig J, Merck E, Mohr F (1837) Das aetherische Oel der Getraide. Ann Pharmacother 24:248–251
2. Eglinton TI, Eglinton G (2008) Molecular proxies for paleoclimatology. Earth Planet Sci Lett 275:1–16
3. Eglinton G et al (1962) Hydrocarbon constituents of the wax coatings of plant leaves: a taxonomic survey. Phytochemistry 1:89–102
4. van Mourik JM, Jansen B (2013) The added value of biomarker analysis in palaeopedology; reconstruction of the vegetation during stable periods in a polycyclic driftsand sequence in SE-Netherlands. Quat Int 306:14–23
5. Jansen B et al (2008) Characteristic straight-chain lipid ratios as a quick method to assess past forest-páramo transitions in the Ecuadorian Andes. Palaeogeogr Palaeoclimatol Palaeoecol 262:129–139
6. Huang Y et al (1996) Isotope and molecular evidence for the diverse origins of carboxylic acids in leaf fossils and sediments from the Miocene Lake Clarkia deposit, Idaho, U.S.A. Org Geochem 24:289–299

7. Bush RT, McInerney FA (2013) Leaf wax *n*-alkane distributions in and across modern plants: implications for paleoecology and chemotaxonomy. Geochim Cosmochim Acta 117:161–179
8. Schwark L, Zink K, Lechterbeck J (2002) Reconstruction of postglacial to early Holocene vegetation history in terrestrial Central Europe via cuticular lipid biomarkers and pollen records from lake sediments. Geology 30:463–466
9. Zheng Y et al (2007) Lipid biomarkers in the Zoigê-Hongyuan peat deposit: indicators of Holocene climate changes in West China. Org Geochem 38:1927–1940
10. Zink K et al (2001) Temperature dependency of long-chain alkenone distributions in recent to fossil limnic sediments and in lake waters. Geochim Cosmochim Acta 65:253–265
11. Grice K et al (2003) Structural and isotopic analysis of kerogens in sediments rich in free sulfurised *Botryococcus braunii* biomarkers. Org Geochem 34:471–482
12. Schouten S et al (2007) Archaeal and bacterial glycerol dialkyl glycerol tetraether lipids in hot springs of Yellowstone National Park. Appl Environ Microbiol 73:6181–6191
13. Schwark L, Empt P (2006) Sterane biomarkers as indicators of palaeozoic algal evolution and extinction events. Palaeogeogr Palaeoclimatol Palaeoecol 240:225–236
14. Yunker MB et al (2002) PAHs in the Fraser River basin: a critical appraisal of PAH ratios as indicators of PAH source and composition. Org Geochem 33:489–515
15. Eckmeier E, Wiesenberg GLB (2009) Short-chain *n*-alkanes (C_{16-20}) in ancient soil are useful molecular markers for prehistoric biomass burning. J Archaeol Sci 36:1590–1596
16. Leythaeuser D, Schwark L, Keuser C (2000) Geological conditions and geochemical effects of secondary petroleum migration and accumulation. Mar Pet Geol 17:857–859
17. Hallmann COE et al (2007) Temporal resolution of an oil charging history – a case study of residual oil benzocarbazoles from the Gidgealpa Field. Org Geochem 38:1516–1536
18. Weijers JWH et al (2010) Carbon isotopic composition of branched tetraether membrane lipids in soils suggest a rapid turnover and a heterotrophic life style of their source organism(s). Biogeoscience 7:2959–2973
19. Wiesenberg GLB et al (2004) Source and turnover of organic matter in agricultural soils derived from *n*-alkane/*n*-carboxylic acid compositions and C-isotope signatures. Org Geochem 35:1371–1393
20. Sachse D, Radke J, Gleixner G (2004) Hydrogen isotope ratios of recent lacustrine sedimentary *n*-alkanes record modern climate variability. Geochim Cosmochim Acta 68:4877–4889
21. Jambu P et al (1991) Incorporation of natural hydrocarbons from plant residues into an hydromorphic humic podzol following afforestation and fertilization. J Soil Sci 42:629–636
22. Cranwell PA (1991) Paleolimnological studies using sequential lipid extraction from recent lacustrine sediment – recognition of source organisms from biomarkers. Hydrobiology 214:293–303
23. Herbin GA, Robins PA (1968) Studies on plant cuticular waxes –I. The chemotaxonomy of alkanes and alkenes of genus *Aloe* (Liliaceae). Phytochemistry 7:239–255
24. Collister JW et al (1992) An isotopic biogeochemical study of the Green River oil shale. Org Geochem 19:265–276
25. Ralph J, Hatfield RD (1991) Pyrolysis-GC-MS characterization of forage materials. J Agric Food Chem 39:1426–1437
26. Beyer L (1996) Soil organic matter composition of spodic horizons in Podzols of the Northwest German Lower Plain. Sci Total Environ 181:167–180
27. Wiesenberg GLB, Schwark L, Schmidt MWI (2004) Improved automated extraction and separation procedure for soil lipid analyses. Eur J Soil Sci 55:349–356
28. Baas M et al (2000) A comparative study of lipids in *Sphagnum* species. Org Geochem 31:535–541
29. Gülz PG (1968) Normale und verzweigte Alkane in Chloroplastenpräparaten und Blättern von *Antirrhinum majus*. Phytochemistry 7:1009–1017
30. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917
31. Peters KE, Walters CC, Moldowan JM (2005) The biomarker guide. Cambridge University Press, Cambridge
32. van Bergen PF et al (1997) Organic geochemical studies of soils from the Rothamsted classical experiments – I. Total lipid extracts, solvent insoluble residues and humic acids from Broadbalk wilderness. Org Geochem 26:117–135
33. McCarthy RD, Duthie AH (1962) A rapid quantitative method for the separation of free fatty acids from other lipids. J Lipid Res 3:117–119
34. Radke M, Willsch H, Welte DH (1980) Preparative hydrocarbon group type

determination by automated medium pressure liquid chromatography. Anal Chem 52:406–411

35. Bianchi G, Corbellini M (1977) Epicuticular wax of *Triticum aestivum* DEMAR 4. Phytochemistry 16:943–945
36. Jetter R, Schäffer S (2001) Chemical composition of the Prunus laurocerasus leaf surface. Dynamic changes of the epicuticular wax film during leaf development. Plant Physiol 126:1725–1737
37. Luque de Castro MD, García-Ayuso LE (1998) Soxhlet extraction of solid materials: an outdated technique with a promising innovative future. Anal Chim Acta 369:1–10
38. Jansen B et al (2006) The applicability of accelerated solvent extraction (ASE) to extract lipid biomarkers from soils. Appl Geochem 21:1006–1015
39. Lüniger G, Schwark L (2002) Characterisation of sedimentary organic matter by bulk and molecular geochemical proxies: an example from Oligocene maar-type Lake Enspel, Germany. Sediment Geol 148:275–288
40. Shantha NC, Napolitano GE (1992) Gas chromatography of fatty acids. J Chromatogr A 624:37–51
41. Wang Z, Yang C, Kelly-Hooper F, Hollebone BP, Peng X, Brown CE, Landriault M, Sun J, Yang Z (2009) Forensic differentiation of biogenic compounds from petroleum hydrocarbons in biogenic and petrogenic compounds cross-contaminated soils and sediments. J Chromatogr A 1216:1174–1191
42. Wakeham SG, McNichol AP (2014) Transfer of organic carbon through marine water columns to sediments – insights from stable and radiocarbon isotopes of lipid biomarkers. Biogeoscience 11:6895–6914
43. Maffei M (1996) Chemotaxonomic significance of leaf wax alkanes in the Gramineae. Biochem Syst Ecol 24:53–64
44. Volkman JK et al (1998) Microalgal biomarkers: a review of recent research developments. Org Geochem 29:1163–1179
45. Lockheart MJ, van Bergen PF, Evershed RP (2000) Chemotaxonomic classification of fossil leaves from the Miocene Clarkia lake deposit, Idaho, USA based on *n*-alkyl lipid distributions and principal component analyses. Org Geochem 31:1223–1246
46. Rommerskirchen F et al (2006) Chemotaxonomic significance of distribution and stable carbon isotopic composition of long-chain alkanes and alkan-1-ols in C_4 grass waxes. Org Geochem 37:1303–1332
47. Harwood JL, Russell NJ (1984) Lipids in plants and microbes. Allen and Unwin, London
48. Maffei M, Badino S, Bossi S (2004) Chemotaxonomic significance of leaf wax *n*-alkanes in the Pinales (Coniferales). J Biol Res 1:3–19
49. Wiesenberg GLB, Schwark L (2006) Carboxylic acid distribution patterns of temperate C_3 and C_4 crops. Org Geochem 37:1973–1982
50. Vogts A et al (2009) Distribution patterns and stable carbon isotopic composition of alkanes and alkan-1-ols from plant waxes of African rain forest and savanna C_3 species. Org Geochem 40:1037–1054
51. Sinninghe Damsté JS et al (2000) Newly discovered non-isoprenoid glycerol dialkyl glycerol tetraether lipids in sediments. Chem Commun 1683–1684
52. Ries-Kautt M, Albrecht P (1989) Hopane-derived triterpenoids in soils. Chem Geol 76:143–151
53. Didyk BM et al (1978) Organic geochemical indicators of palaeoenvironmental conditions of sedimentation. Nature 272:216–222
54. Weijers JWH et al (2006) Occurrence and distribution of tetraether membrane lipids in soils: implications for the use of the TEX_{86} proxy and the BIT index. Org Geochem 37:1680–1693
55. Garcin Y et al (2012) Hydrogen isotope ratios of lacustrine sedimentary *n*-alkanes as proxies of tropical African hydrology: insights from a calibration transect across Cameroon. Geochim Cosmochim Acta 79:106–126
56. Huang Y et al (2004) Hydrogen isotope ratios of individual lipids in lake sediments as novel tracers of climatic and environmental change: a surface sediment test. J Paleolimnol 31:363–375
57. Chikaraishi Y, Naraoka H (2007) $\delta^{13}C$ and δD relationships among three *n*-alkyl compound classes (*n*-alkanoic acid, *n*-alkane and *n*-alkanol) of terrestrial higher plants. Org Geochem 38:198–215
58. Cayet C, Lichtfouse É (2001) $\delta^{13}C$ of plant-derived *n*-alkanes in soil particle-size fractions. Org Geochem 32:253–258
59. Lichtfouse É et al (1997) Molecular, ^{13}C, and ^{14}C evidence for the allochthonous and ancient origin of C_{16}-C_{18} *n*-alkanes in modern soils. Geochim Cosmochim Acta 61:1891–1898
60. Rethemeyer J et al (2004) Complexity of soil organic matter: AMS ^{14}C analysis of soil lipid fractions and individual compounds. Radiocarbon 46:465–473
61. Bol R et al (1996) The ^{14}C age and residence time of organic matter and its lipid constituents in a stagnohumic gley soil. Eur J Soil Sci 47:215–222

62. Jenkins BM et al (1996) Emission factors for polycyclic aromatic hydrocarbons from biomass burning. Environ Sci Technol 30:2462–2469

63. Simoneit BRT, Elias VO (2000) Organic tracers from biomass burning in atmospheric particulate matter over the ocean. Mar Chem 69:301–312

64. Birk JJ et al (2012) Combined quantification of faecal sterols, stanols, stanones and bile acids in soils and terrestrial sediments by gas chromatography–mass spectrometry. J Chromatogr A 1242:1–10

65. Evershed RP (2008) Organic residue analysis in archaeology: the archaeological biomarker revolution. Archaeometry 50:895–924

Author Queries

Chapter No.: 157

Query Refs.	Details Required	Author's response
AU1	Please provide the siginificance of footnote "1“ in the sectence "If possible, plastics should be...	
AU2	Please clarify the uage of ff along with section number here and in subsequent occurrences.	